REMARKS

Status of Claims

Claims 21-29 are pending in the application. Claims 21-29 have been rejected.

35 U.S.C. § 112 Rejections

In the Office Action, the Examiner maintained his rejection of claims 21-29 under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. Applicants disagree.

The claims are directed to a method of inducing an immune response against a cancer cell in a mammal via administration of an auxotrophic attenuated strain of *Listeria* comprising a heterologous (cancer cell) antigen. Applicants have demonstrated possession of the claimed invention by exemplifying a method of inducing an immune response in a mammal using a particular auxotrophic *Listeria* as an example (see paragraphs 0106-0110). Applicants have also provided written description of other auxotrophic mutations (see paragraph 0040), a description of how to generate auxotrophic mutants and methods to test for their auxotrophic phenotype (see paragraphs 0037-0039, 0049-0057, 0079-0088, and 0092-0097), and methods to test for the immunogenicity of auxotrophic *Listeria* strains (see paragraphs 0057-0058 and 0090-0091). In addition, auxotrophic mutants were known in the art, and the level of skill in the art of microbiology and specifically *Listeria* microbiology is very high, such that the generation of mutants is considered routine in the art and (Marquis et al, Infect Immun. 1993 Sep;61(9):3756-60; Camilli et al, J. Bacteriol. 1990 Jul;172(7):3738-44 attached

hereto; Alexander et al. Infect Immun 61.5 (1993): 2245-8, attached hereto). Therefore, it would be clear to a person skilled in the art that the inventors had possession of the claimed invention.

The Examiner alleged that "the disclosure fails to describe the generation of other suitable strains and fails to provide a reproducible means for obtaining said strains." As described above, Applicants provide numerous methods in the specification that would allow a person with ordinary skill in the art to generate auxotrophic *Listeria* strains.

The Examiner further alleged that the subject specification does not provide support for other strains that are attenuated and highly immunogenic. However, examples of such strains were known in the art at the time of filing (for e.g., Alexander et al.). In addition, the level of skill and knowledge in the art at the time of filing would have allowed a person skilled in the art to generate auxotrophic attenuated strains that are highly immunogenic for use as live vaccine vehicles, as was demonstrated by Alexander et al. Examples of methods that a person of ordinary skill in the art may use to render the auxotrophic strains more immunogenic are disclosed in the subject specification (paragraphs 0061-0063).

The Examiner also alleged that the disclosure appears to suggest that double mutants are required to practice the invention. Applicants disagree. *Listeria* strains comprising a mutation in at least one gene whose protein product is essential for growth of attenuated *L. monocytogenes* strains that can be used as vaccine vehicles are disclosed in the subject specification (see paragraphs 0038 and 0040), in the references described *supra*, and in other references (for example, Rouquette et al. FEMS Microbiol Lett 133.1-2 (1995): 77-83 attached hereto). Thus, it is clear that a double mutant is not necessary in all cases to obtain an auxotrophic mutant and/or to practice the invention.

Applicants thank the Examiner who appears to have accepted Applicants' arguments from the response to office action dated March 22, 2007 that a person skilled in the art would be able to substitute the attenuated auxotrophic strains known in the art, such as those described by Camilli et al. and Marquis et al. for the auxotrophic strains described in the subject specification, as the Examiner has not provided a rebuttal.

The Examiner maintained his rejection of claims 21-29 under 35 U.S.C. § 112, first paragraph, alleging that the specification does not enable a person skilled in the art to make and/or use the invention commensurate in scope with the claims. Applicants disagree. Working examples for making and using attenuated auxotrophic mutant strains (see paragraphs 0107-0111) are provided, including detailed methodology to curtail the time required to carry out the generation of attenuated auxotrophic *L. monocytogenes* mutants (see paragraphs 0037-0039 and 0049-0057 and 0061-0063), providing the necessary amount of direction or guidance for a skilled artisan to generate auxotrophic mutants. Further, the state of the art and relative skills of those in the art at the time of filing would have enabled a person skilled in the art to generate other attenuated auxotrophic mutants (see, for example, Alexander et al. Infect Immun 61.5 (1993): 2245-8). Thus, based on the Wand's factors, the breadth of the claims is fully supported by the specification as filed based on the knowledge in the art at the time of filing.

The Examiner conceded that while it is possible to generate additional auxotrophic mutants, it is allegedly unclear which of these mutants will remain sufficiently attenuated to not cause disease, but still allow expression and presentation of the immunogen of interest. Again, the specification describes methods of evaluating attenuated *Listeria* (see paragraphs 0037-0039, 0049-0057, 0079-0088, and 0092-0097),

of evaluating immunogenicity of *Listeria* (paragraphs 0057-0058 and 0090-0091), and examples of such attenuated, immunogenic strains were presented in the specification (paragraphs 0106-0110) and were known in the art (Alexander et al.). Thus, it would not entail undue experimentation to generate, identify, and use appropriate auxotrophic mutants.

The Examiner alleged that the disclosure fails to describe the generation of other suitable strains or to provide a single working embodiment other than the dal⁻/dat⁻ double mutant. Applicants respectfully disagree and assert that the specification provides an enabling description of auxotrophic attenuated strains of *Listeria* other than dal and dat strains and their generation (paragraphs, 009, 0037, and 0040), thus describing the generation of other suitable strains.

The Examiner alleged that Marquis et al. (Infect Immun 61.9 (1993): 3756-60) and Portnoy et al. (US-Patent No. 5830702. November, 3. 1998) allegedly describe challenges involved in generating attenuated, auxotrophic *Listeria* mutants with the desired biological properties. More specifically, the Examiner indicated that Portnoy et al. allegedly states that certain nutritional auxotrophs may not be easily attenuated, and that Marquis et al. notes that transposon insertion auxotroph mutants were virulent and grew similarly to the parental strain and that the intracellular milieu of eukaryotic cells is a nutritious niche that allows the propagation of *Listeria* mutants. However, the specification (see paragraphs 0102 and 0103 and 0037) and the prior knowledge in the art (Alexander et al.) clearly report success in generating auxotrophic attenuated mutants that are avirulent and are used to protect the host from *L. monocytogenes*-related disease. Applicants note that it is not necessary that every permutation within a generally operable invention be effective in order for an inventor to obtain a generic claim, provided that the

effect was sufficiently demonstrated to characterize a generic invention. See Capon v. Eshar (418 F 3d 1349 (Fed Cir 2005)). In this case, the method exemplified in dal/dat mutants would certainly lead a skilled artisan to use the method in other auxotrophic mutants.

In view of the foregoing arguments, Applicants respectfully assert that claims 21-29 are proper under 35 U.S.C. 112 and respectfully request that the rejections be withdrawn. Accordingly, the full scope of the invention as recited in the subject claims is fully described and enabled in the subject specification.

Should the Examiner have any question or comment as to the form, content or entry of this Amendment, the Examiner is requested to contact the undersigned at the telephone number below. Similarly, if there are any further issues yet to be resolved to advance the prosecution of this application to issue, the Examiner is requested to telephone the undersigned counsel.

Please charge any fees associated with this paper to deposit account No. 50-3355

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An iron-dependent mutant of *Listeria monocytogenes* of attenuated virulence

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Abstract

A bank of Tn*917*-insertional mutants from the facultative intracellular pathogen *Listeria monocytogenes* was screened by an original method based on bacterial growth on synthetic medium under iron-limiting conditions. One mutant, whose in vitro growth in synthetic medium was specifically dependent upon the availability of iron in its environment, was isolated and characterized. The insertional event occurred in a non-coding region, upstream of a *rrn* operon and located within a 1100-kb *Not*I fragment of the physical map, where the virulence genes already identified in *L. monocytogenes* were also present. Protein analysis by SDS-PAGE revealed a pleiotropic effect of the insertional event on cell-associated proteins, suggesting a polar effect of the transposon on adjacent unknown gene(s). The virulence in the mouse of this mutant was strongly impaired, although it was capable in vitro of growing intracellularly and of spreading from cell to cell, as shown by the production of lytic plaques on cell culture.

Keywords: *Listeria monocytogenes*; Virulence; Iron

1. Introduction

Iron is essential for bacterial growth, serving as a catalyst in electron transport processes required for respiration [1]. Bacteria exposed to iron-limiting conditions have elaborated a large array of mechanisms to acquire free iron from the environment, especially bacterial pathogens [2]. Indeed, the concentration of free iron Fe^{3+} in animal tissues does not exceed about 10^{-18} M, because iron is complexed to various molecules, including heme and proteins, as lactoferrin and transferrin in the extracellular fluids or ferritin and iron-sulfur proteins in the intracellular environment [1]. This amount is not sufficient to support microbial growth, since Gram-negative bacteria require 0.3–1.8 μM and Gram-positive bacteria and fungi 0.4–4 μM iron [3]. The limiting level of free iron in vivo therefore restricts the multiplication of most microorganisms encountered in nature, except microbial pathogens that replicate in host tissues during the infectious process [2]. Although our knowledge on the mechanisms of iron acquisition systems in vitro have greatly progressed during the recent years, their relevance to the in vivo setting remains poorly understood, especially with regard to intracellular pathogens.

Listeria monocytogenes is the prototype of facultative intracellular pathogens, responsible for severe

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infections in most animal species, including humans. The expression of virulence of this pathogen is dependent upon the in vivo level of iron, since administration of iron in mice increases its virulence, whereas this can be reversed by removing host iron with chelating agents [4]. Moreover, the intracellular amount of iron regulated through transferrin receptors is essential for the bactericidal activity of macrophages and for intracellular growth of *L. monocytogenes* [5]. Several reports have studied the mechanisms of iron acquisition in vitro by *L. monocytogenes* [6–9]. This pathogen does not produce siderophores, but instead releases a soluble, low molecular mass (8–10 kDa) reductant that removes Fe^{3+} from transferrin and produces Fe^{2+}, allowing direct interaction of Fe^{2+} with bacterial surface binding sites [6]. This extracellular reductase requires NADH, flavin mononucleotide and Mg^{2+} as cofactors [7,8]. Other iron acquisition systems have been described, involving direct binding of ferric citrate on the cell surface [7] and direct binding of transferrin through a specific 126-kDa receptor [9]. However, nothing is known about the genetics of these iron acquisition systems and their role in vivo during the infectious process. In this work, we isolated an iron-dependent, Tn*917* insertional mutant from *L. monocytogenes* expressing attenuated virulence.

2. Materials and methods

2.1. Bacterial strains and transposon mutagenesis

Listeria monocytogenes LO28, a virulent hemolytic strain was used in this study. Mutagenesis was performed with transposon Tn*917* present on plasmid pTV1, as previously described [10]. Bacteria were grown to mid-exponential phase at 30°C in BHI broth with chloramphenicol (7 μg ml^{-1}) and erythromycin (5 μg ml^{-1}). Tn*917* insertion mutants were obtained by incubating bacteria 48 h at 42°C on BHI agar supplemented with the same concentration of erythromycin, thus selecting for transposition events from the plasmid into the chromosome and for loss of the plasmid pTV1. The transposon-induced mutants were shown to be chloramphenicol-sensitive, plasmid-free. The transposition events occurred at a frequency of 10^{-5}. Each mutant was subcultured in BHI broth (erythromycin 10 μg ml^{-1}) on microplates incubated for 24 h at 37°C and then stored at −80°C.

2.2. Culture media and bacterial growth curves

Bacteria were routinely grown on complex culture media, as Brain-Heart-Infusion (BHI) agar and broth (Diagnostics Pasteur, Marnes-la-Coquette, France), trypticase-soya agar supplemented with 1% (w/v) glucose, and Columbia agar medium supplemented with 5% (v/v) horse blood (bioMérieux, Marcy-l'Etoile, France). Biochemical identification was made on API-50 CH (bioMérieux). Hemolysin titration and lecithinase production were performed on culture supernatants of bacteria grown in BHI broth, as previously described [11]. Anaerobic cultures were made on blood agar under anaerobic conditions (GasPak, bioMérieux). A synthetic medium derived from that described by Ralovitch et al. [12] was also used. The base medium designated F0 (2×) was composed as follows: KH_2PO_4, 9 g; K_2HPO_4, 21 g; NaCl, 10 g; $(NH_4)_2SO_4$, 42 g; glucose, 40 g; L-isoleucine, 100 mg; L-leucine, 100 mg; L-valine, 100 mg; L-arginine, 200 mg; L-glutamine, 100 mg; L-histidine, 200 mg; L-methionine, 100 mg; riboflavin, 8 mg; biotin, 8 mg; thiamine, 20 mg; $MgSO_4 \cdot 7H_2O$, 400 mg; $CuCl_2 \cdot 6H_2O$, 1.8 mg; thioctic acid, 20 mg; cysteine, 0.4 mg; in a volume of 1 l. Cysteine was added before use. All products were obtained from Sigma Chemical Co. and Prolabo (Paris, France). This synthetic liquid medium F0 contained less than 1 μM of iron and was supplemented in a volume of 1 l with double-distilled water (v/v) or with solution (14 g l^{-1} final, v/v) agar (Agar Noble, Difco Laboratories, Detroit, MI), and eventually added with increasing concentrations of $FeSO_4$ (Sigma) at pH 7.2. $FeSO_4$ is easier to dissolve at neutral pH than $Fe_2(SO_4)_3$ or $FeCl_3$. We also used $FeCl_3$ (Sigma) to complement the synthetic media with the same results. According to the final concentration of $FeSO_4$, these media were designated F35 (35 μM), F70 (70 μM) and F350 (350 μM). F0 was depleted of iron with a chelator, ethylene diamine *N,N*-diacetic acid (EDDA, Sigma). LO28 could grow in liquid or solid synthetic medium F0 added with up to 150 μM EDDA (final). This iron-depleted medium (designated E150) was used to screen iron-dependent mutants from LO28. Synthetic media were always inoc-

ulated with bacteria grown on iron-limiting conditions incubated for 48 h at 37°C. All bacterial cultures in synthetic media were made with plastic flasks and iron-free materials. Growth curves of mutants from LO28 were followed after incubation of bacteria in shaking water bath at 37°C on various synthetic media supplemented with erythromycin (10 μg ml^{-1}). LO28 was cultured in antibiotic-free medium. At various times, aliquots were adequately diluted and plated on BHI agar (bioMérieux) and colonies were counted after a 24-h incubation. The generation time was estimated by calculating the average slope of the bacterial replication curve during the exponential growth phase.

2.3. Chemicals, enzymes, DNA techniques

Restriction enzymes were purchased from New England Biolabs inc. (Beverly, MA) and were used as prescribed by the manufacturer. *Listeria* chromosomal DNA, DNA electrophoresis, Southern blotting and hybridization with ^{32}P-labelled probes were performed as previously described [10,13]. Cloning was done in pUC18 using *Escherichia coli* MC1061 [13]. The nucleotide sequence was determined by dideoxy-chain termination method with the modified T_7 DNA polymerase sequenase™ (United States Biochemical Corp.), and [α-^{35}S]dATP. Pulsed-field gel electrophoresis (PFGE) was performed on chromosomal DNA of *L. monocytogenes* as previously described [14]. DNA samples in agarose were then digested with 10 U of *Not*I (Biolabs) at 37°C. Restriction fragments were separated by PFGE using a CHEF-DRII apparatus (Bio-rad). After electrophoresis, DNA was transferred to Hybond N filter (Amersham) as previously described [14]. Southern blot hybridization was performed using a *erm* probe obtained from Tn*917* harbored on pTV1 [15]. The probe was labelled with digoxigenin-11-dUTP and detected with the Non-radioactive labelling and detection kit (Boehringer Mannheim Biochemicals, Tutzing, Germany).

2.4. Iron reductase activity

The plaque assay for ferric iron reducing activity was as modification of the method of Deneer and Boychuk [8]. The bacterial suspension in distilled water (5 μl) was plated on TS agar with or without ferric ammonium citrate (1 mg ml^{-1}) and ethylenediamine-di (*O*-hydroxyphenylacetic acid) (EDDHA) (1.3 mM). After overnight incubation at 37°C, plates were overlaid with 0.5% low melting point agarose solution containing $MgCl_2$ (10 mM), NADH (15 μM), flavin mononucleotide (FMN, 3 μM) and the ferrous iron chelator ferrozine (2 mM) which visualizes the reductase activity as a purple color [8]. *E. coli* MC1061 was used as a negative control.

2.5. SDS-PAGE and Western blot analysis

SDS polyacrylamide gel (10%) electrophoresis was performed on washed bacteria as previously described [11]. Proteins were detected by Coomassie brilliant blue (Bio-rad). Western blot analysis was performed on supernatants from bacteria grown for 9 h at 37°C in 2 mM EDDA-BHI broth, as previously described [11], using a rabbit anti-LLO [11] and revealed with a peroxidase-labelled goat anti-rabbit immunoglobulin (Organon Teknika, Cappel, Malvern, PA), diluted 1:1000. Enzymatic activity was revealed by the addition of diaminobenzidine-tetrahydrochloride (Sigma) supplemented with hydrogen peroxide (0.02%).

2.6. Plaque assays on cell monolayers and virulence in the mouse

Plaque assays on HEp-2 cells were performed as previously described [16], except that infection was made with various inocula from strains in the exponential growth phase (10^4, 10^5 or 10^6 bacteria per well). For virulence tests, specific-pathogen-free ICR female Swiss mice (Charles River, St. Aubin-les-Elbeuf, France), 6–8 weeks old, were inoculated intravenously with bacteria grown in BHI. Virulence was estimated by monitoring bacterial growth in liver and spleen at various times after infection, as previously described [10].

3. Results and discussion

3.1. Isolation of a Tn917-induced iron-dependent mutant from L. monocytogenes

A bank of 3200 Tn*917*-insertional mutants was constituted by using LO28-pTV1 and stored on mi-

croplates at −80°C. For screening, 10 μl of each well containing individual mutants were subcultured on liquid synthetic medium F35 supplemented with erythromycin (10 μg ml^{-1}) and incubated for 3 days at 37°C. This step of bacterial growth was crucial to partially deplete iron from the bacterial inoculum grown on BHI broth and to allow further screening procedures. All mutants were grown up to 5×10^7 bacteria ml^{-1} and then screened on two liquid synthetic media containing erythromycin (10 μg ml^{-1}): F350 (iron-supplemented medium) and E150 (iron-depleted medium). Out of 3200 mutants, only seven mutants were capable of growing on F35 and none on E150, as opposed to the parental strain LO28 which could grow on both media. One iron-dependent mutant, designated 3H12, was stable and was further characterized, as compared to LO28 and to a randomly chosen control, Tn*917*-insertional mutant 19E8, also capable of growing on both screening media as well as the parental strain. The bacterial growth of 3H12 was first studied on BHI broth at 37°C in a shaking water bath as compared to LO28 and mutant 19E8. No difference in bacterial multiplication was observed between the strains, with a mean generation time estimated at about 0.7 h (data not shown). Growth of 3H12 was then tested on liquid synthetic media, F0, F70, and F350, after inoculation with bacteria grown 48 h in F70 (Fig. 1). Bacterial growth of LO28 was restricted on synthetic media, with a mean generation time estimated at 4.2 h on F0. Similar results were obtained with 19E8 (not shown). In contrast, growth of 3H12 was severely inhibited on F0 with a mean generation time of 17.5 h. Bacterial multiplication was restored in 3H12 by addition of iron reaching 4.5 h in F70 (as compared to 3.1 h for LO28 and 19E8), and 3.3 h in F350, as the control strains. Moreover, bacterial growth curves of mutants were also determined in synthetic media added with $FeCl_3$ with the same results. These results confirm that bacterial replication of 3H12 is strictly dependent upon the availability of iron.

3.2. *Phenotypic analysis of the iron-dependent Tn917 mutant*

Mutant 3H12 was only resistant to erythromycin and harbors a single copy of Tn*917* as previously

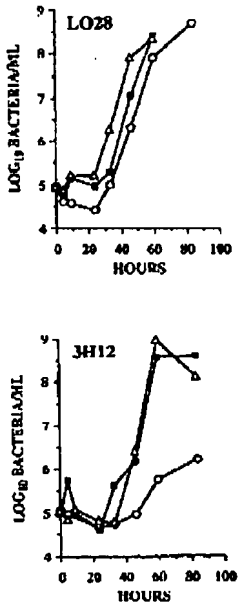

Fig. 1. Bacteria (mutant 3H12 or strain LO28) were grown on liquid synthetic medium F0 (○), or on the same medium complemented with iron ($FeSO_4$), either F70 (■) or F350 (△). Bacteria were counted by plating on BHI agar at progressive time. Bacterial growth of 3H12 was strongly restricted on F0, as compared to LO28, and restored by addition of iron.

described [10,15]. It did not differ from LO28 and 19E8 with respect to its microscopic morphology, colonial aspect, motility, catalase, lecithinase and biochemical tests on API-50 CH. No difference in hemolysin production on blood agar medium in aerobic and anaerobic conditions. Hemolytic titers in supernatants of BHI broth (10 h–18 h) were similar to those of control strains, as confirmed by Western blot analysis of supernatants using an anti-LLO serum (not shown). The iron reductase activity of 3H12 was not inhibited since the mutant could reduce ferric iron (as LO28 and 19E8), giving rise to purple colonies on specific medium.

The protein pattern of 3H12 was studied by SDS-PAGE with lysates of washed bacteria grown on BHI broth. As illustrated in Fig. 2, several differences were consistently detected in these conditions, especially the presence of additional bands (84–97 kDa), and the loss of two bands (22–25 kDa), as

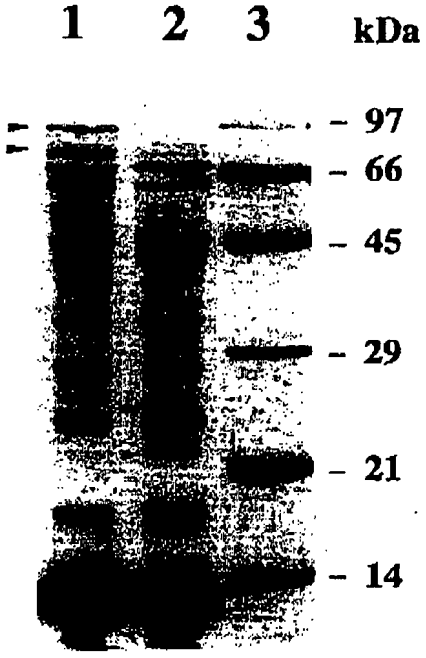

Fig. 2. SDS-PAGE analysis of bacterial lysates from washed bacteria of mutant 3H12 (lane 1) and LO28 (lane 2). Lane 3, molecular size markers. Several differences were detectable, especially the presence of two additional bands (84–97 kDa) and the loss of two bands (22–25 kDa), as indicated by arrows. The single insertional event has a pleiotropic effect in mutant 3H12.

compared to the protein patterns of LO28 (Fig. 2). Mutant 19E8 displayed the same pattern as LO28 (not shown). The same results were obtained after growth on synthetic medium F0 (data not shown). No difference was detected by SDS-PAGE analysis of the supernatants (data not shown), indicating that the iron-dependent phenotype might be related to cell-associated proteins rather than to secreted proteins. This pleiotropic effect on the protein pattern as a result of a single insertional event favors the view that the transposon inhibits a gene or a set of genes acting on multiple targets, such as regulatory genes that might control the iron-dependent phenotype.

3.3. *Tn917 has inserted in a non-coding region downstream of a rrn operon*

The next step was to locate the site of the transposon insertion on the chromosomal DNA of *L. monocytogenes*. For this purpose, the flanking region of the transposon insertion on the chromosomal DNA of 3H12 was cloned into the *Hin*dIII site of pUC18. After transformation of *E. coli* MC1061, transformant *E. coli* were selected for erythromycin resistance, the *erm* gene being located at the left end of Tn*917* without the *Hin*dIII site, thus generating pAB21. The sequence analysis of the 3.14-kb insert of pAB21 revealed that Tn*917* has inserted into a non-coding region of the chromosomal DNA (Fig. 3). Interestingly, at the left part of the 208-bp fragment of chromosomal DNA, there is a 62-bp region showing 96% nucleotide homology with the gene coding for the 5S RNA of *Bacillus methanolicus* and related Gram-positive bacilli belonging to the genus *Bacillus* (the 5S rRNA gene of *L. monocytogenes* has not been previously sequenced). By cloning a 5-kb *Eco*RI fragment from the chromosomal DNA of LO28 using a 214-bp *Hin*dIII–*Aca*I probe from pAB21, we sequenced a 502-bp fragment located downstream the 5S RNA gene, which shares almost

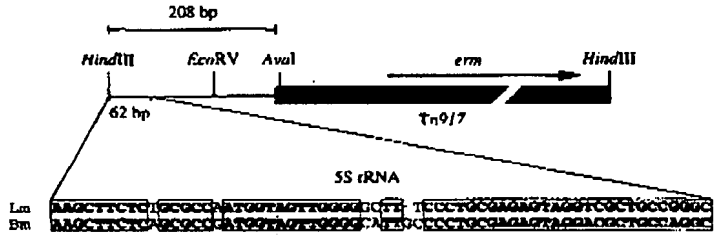

Fig. 3. Schematic map of the *Tn*917 insertional region of mutant 3H12. A 62-bp sequence located at the left part of the chromosomal DNA shares 96% nucleotidic identity with the gene coding for the 5S RNA of *Bacillus methanolicus* (Lm, *Listeria monocytogenes*; Bm, *Bacillus methanolicus*).

100% nucleotide homology with the 23S rRNA gene of *L. monocytogenes* previously sequenced [17,18], thus indicating the left orientation of the *rrn* operon. We then located the insertion of Tn*917* on the chromosomal DNA by PFGE. The DNA fragments from 3H12 and LO28 were probed with an intragenic *erm* probe, obtained from pTV1. It was found by Southern blotting that the probe recognized only a large *Not*I fragment of 1100 kb previously identified as containing the virulence genes, including the virulence operons *prfA* and lecithinase, and the *inlA–inlB* genes [14]. Our results indicate that transposon insertion took place upstream one of the six *rrn* operons described in *L. monocytogenes* [14]. The pleiotropic effect on the protein pattern of the mutant, as a result of a single insertional event, strongly suggests a polar effect on adjacent unknown genes located at the right of the transposon. By colony hybridization using the 214-bp *Hin*dII–*Ava*I fragment from pAB21 as a probe, we succeeded in cloning a 5-kb region of LO28 where Tn*917* had inserted. Sequencing of this entire region currently in progress shows that Tn*917* has inserted upstream of at least three ORFs oriented in the opposite direction of the *rrn* operon.

3.4. Iron-dependent mutant has attenuated virulence

Mutant 3H12 was able to invade and grow in vitro in HEp-2 cells, producing lytic plaques of the same size and number as those observed with the parental strain, indicating that 3H12 can invade, multiply inside host cells and spread from cell to cell, as LO28. The virulence of bacterial strains (3H12, LO28, and 19E8) was then studied in Swiss mice by determining the kinetics of bacterial growth in the spleen and liver after i.v. inoculation of 5×10^5 bacteria per mouse (Fig. 4). LO28 and 19E8 rapidly grew in organs until the death of mice by day 3, reaching 10^7–10^8 per organ. The early kinetics of bacterial multiplication of 3H12 was very similar to those of control strains in the spleen and liver during the first 24 h of infection (Fig. 4), suggesting that bacteria were capable of invading and multiplying in hepatocytes and macrophages. After 24 h, the growth of mutant 3H12 was curtailed, with a rapid elimination of bacteria from the spleen and liver by day 2–5 of infection.

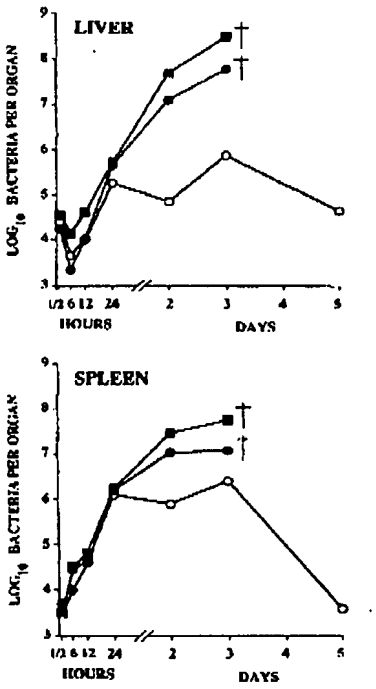

Fig. 4. Virulence in the mouse of mutant 3H12. Mice were inoculated i.v. with 5×10^5 bacteria of various strains (mutant 3H12, ○; LO28, ■; mutant 19E8, ●). Bacterial growth was then followed in the spleen and liver. The early kinetics of bacterial growth of 3H12 were very similar to those of control strains during the first 24 h of infection. Subsequently, bacterial growth of the mutant was reduced, thus resulting in the progressive elimination of bacteria from the organs. In contrast, mice infected by LO28 and 19E8 died by day 4, with a rapid unrestricted multiplication in organs.

The finding that 3H12 produced lytic plaques in vitro on confluent HEp-2 cells confirms that the *prfA*-dependent virulence genes implicated in the intracellular cycle are fully expressed in 3H12. The reason why bacterial growth is curtailed in vivo after 24 h of infection, at a time when bacteria are released from hepatocytes due to the in situ recruitment of cytotoxic cells, including neutrophils [19], remains to be determined. However, it must be considered that the intracellular milieu contains a variety of potential iron sources readily available to intracytoplasmic pathogens, including heme, heme proteins, the iron storage protein ferritin, iron–sulfur proteins

and a small pool of free ferrous iron. Therefore, it is possible that there is no strong iron limitation in the intracellular environment for intracytoplasmic bacteria, especially inside hepatocytes, which means that the curtailment of bacterial growth in vivo might mainly take place as soon as bacteria are released from their intracellular sanctuary. Whether this restriction of bacterial growth is directly due to the iron-dependent phenotype or to other unknown factors that might be under the same control of putative regulatory gene(s) abrogated by the transposon insertion, remains to be determined by the entire sequencing of the insertional region which is currently in progress.

Acknowledgements

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Characterization of an Aromatic Amino Acid-Dependent *Listeria monocytogenes* Mutant: Attenuation, Persistence, and Ability To Induce Protective Immunity in Mice

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A transposon insertion mutant of *Listeria monocytogenes* was shown to be deficient in prephenate dehydratase, an enzyme acting late in the pathway for biosynthesis of phenylalanine. This mutant had reduced virulence in mice. The mutant and parent strains persisted to the same extent in the tissues of infected mice and elicited similar degrees of splenomegaly. Mice vaccinated with the mutant were protected significantly from subsequent challenge with virulent *L. monocytogenes*.

In farm animals, especially sheep, losses due to deaths from listeriosis can be considerable (21). To protect against infection, killed and live attenuated strains have been used as vaccines in animals, but with little or no success (1, 3, 8, 13). The failure of previous live attenuated strains as vaccines is probably due to the method of attenuation used (8, 13).

An alternative to the procedures previously used for making attenuated strains is the generation of strains with a defined mechanism of attenuation. This approach is the basis of the suggestion that hemolysin-deficient strains of *Listeria monocytogenes* could be suitable as attenuated strains for vaccines (15). However, use of these strains would exclude protection from the effects of hemolysin, an important virulence factor (18). Therefore, we decided to assess the potential of aromatic amino acid-dependent, hemolytic strains of *L. monocytogenes* for use as live vaccines. Aromatic amino acid-dependent (*aro*) strains of *Salmonella typhimurium* and *Salmonella typhi* are very successful examples of the use of defined mutants as vaccines (12, 17, 19) against salmonellosis. We assessed an aromatic amino acid-dependent transposon insertion mutant of *L. monocytogenes* as a candidate vaccine in a mouse model of listeriosis.

L. monocytogenes Lm.918.6 is an aromatic amino acid-dependent mutant of the wild-type strain Lm.1070138 (2). Both strains were obtained from D. Portnoy, University of Pennsylvania, and maintained as described before (20). When necessary, minimal medium (6), supplemented with the appropriate amino acid at 20 μg/ml, was used. Cells for inoculation into mice were grown at 30°C to late log phase in tryptose soya broth (Difco), supplemented when appropriate with erythromycin (25 μg/ml). The cells were then resuspended in tryptose soya broth containing 10% (vol/vol) glycerol and stored at −20°C for several months without significant loss of viability. When required, the bacteria were thawed rapidly, harvested by centrifugation, and resuspended in sterile distilled water. The specific activity of prephenate dehydratase was assayed by the method of Nester and Jensen (16). Female MF1 outbred mice, ca. 30 g in weight (Harlan Olac Ltd., Shaw's farm, Bicester, United Kingdom), were used throughout. Virulence was estimated by determining the 50% effective dose (ED_{50}). Doses of bacteria between 10^3 and 10^8 CFU in 100 μl of distilled water were administered intravenously to groups of five mice. The number of mice per group that reached the end point of the assay (i.e., became moribund) between days 0 and 6 was recorded and used to calculate the ED_{50} by the log-probit method (the percentage of mice surviving to end point versus the log of the CFU inoculated) (5).

In experiments to ascertain the numbers of bacteria in the livers and spleens, infected mice were killed by cervical dislocation at the desired times after infection. The spleens and livers were removed, weighed, and homogenized separately in 10 ml of sterile distilled water in a Stomacher-Lab blender (Seward Medical), and viable counts on tryptose agar were obtained (20). Results were expressed as mean counts of viable listeriae per gram of tissue. To determine whether vaccination with Lm.918.6 conferred resistance to subsequent infection, mice were first vaccinated (intravenously) with 10^4 CFU of Lm.918.6 in water. The booster vaccination was an identical dose of Lm.918.6 given 14 days after the initial vaccination. At 28 days after the initial vaccination with Lm.918.6, the ED_{50} of Lm.1070138 for the mice was determined as described above. Data were analyzed by the Mann-Whitney U test (10) or the Kolmogorov-Smirnov test (10).

Lm.918.6 had been described previously as a Tn*917* insertion mutant that required aromatic amino acids for growth (2). To ensure that Lm.918.6 contained a single Tn*917* insertion, a Southern blot was performed to *Eco*RI-digested chromosomal DNA with a radiolabelled Tn*917*-specific probe as described in reference 2. Uncut plasmid pTV32 was included as a positive control, as it carries a single copy of Tn*917* (22). The probe hybridized to a single *Eco*RI fragment (Fig. 1). Since there are no recognition sites for *Eco*RI within Tn*917* (2), Lm.918.6 appears to contain a single Tn*917* insertion. To characterize more precisely the biochemical defect, aromatic amino acids and intermediates in the aromatic amino acid pathway were assayed for their ability to support the growth of Lm.918.6 in minimal medium. While phenylalanine and its immediate precursor, phenylpyruvic acid, supported growth, neither tyrosine nor prephenate (a precursor of both tyrosine and phenylpyruvic acid) did. To explain this, the activity of prephenate dehydratase, the enzyme that catalyzes the conversion of pre-

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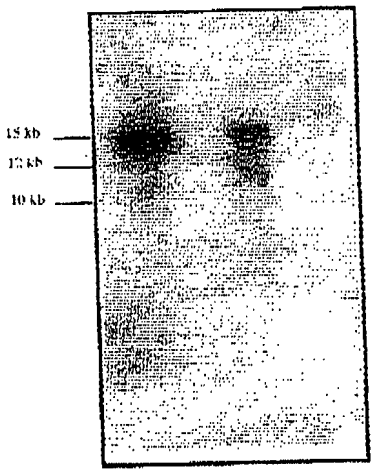

FIG. 1. Southern blot of *Eco*RI-digested Lm.918.6 chromosomal DNA (a) and uncut plasmid pTV32 DNA (b), probed with a 1.5-kb transposon Tn*917*-specific DNA probe (2). Lambda DNA digested with either *Xho*1 or *Hin*dIII was used as molecular weight markers.

phenic acid to phenylpyruvic acid, in both Lm.1070138 and Lm.918.6 was determined. In Lm.918.6, the activity was 0.02 U/mg of protein, whereas the wild-type level was 7.00 U/mg of protein. This suggested that the single transposon insertion in Lm.918.6 was affecting expression of the *pheA* gene, which encodes prephenate dehydratase. As measured by ED_{50}, strain Lm.918.6 was significantly ($P < 0.05$) attenuated compared with the wild-type strain, Lm.1070138, in the mouse model of listeriosis (Table 1). Lm.918.6 multiplied to significantly ($P < 0.05$) lower numbers in both the liver and the spleen than Lm.1070138 (Fig. 2A and B). After a dose of 10^4 viable cells of Lm.1070138, peak counts of 6×10^9 CFU/g of spleen tissue and 7×10^7 CFU/g of liver tissue were obtained after 3 days. At the same dose, Lm.918.6 grew more slowly, reaching peak counts of 2×10^6 and 9×10^5 CFU/g of spleen and liver tissue, respectively, at day 5 (Fig. 2A and B). No significant difference ($P > 0.05$) in the clearance of the two strains between days 1 and 9 was noted, and by day 11, both strains had been cleared (Fig. 2A and B). Maximum splenomegaly, as measured by the mean spleen weight of the five animals at each time point, occurred 4 days after maximum numbers of listeriae were detected, i.e., on day 7 postinfection with the wild-type strain Lm.1070138 and on day 9 postinfection with Lm.918.6 (Fig. 2C). The extent of splenomegaly induced by Lm.918.6 was not significantly

TABLE 1. Comparison of ED_{50}s of Lm.1070138 and Lm.918.6

Strain	ED_{50} (CFU)		
	Mean[a]	SEM	95% CL[b]
Lm.1070138	5.51×10^4	4.97×10^2	1.28×10^4–2.37×10^5
Lm.918.6	$2.61 \times 10^{6*}$	3.67×10^5	7.00×10^5–9.71×10^6

[a] Mean dose required to render 50% of the mice moribund. *, significantly different ($P < 0.05$) from value for Lm.1070138.
[b] CL, confidence limits.

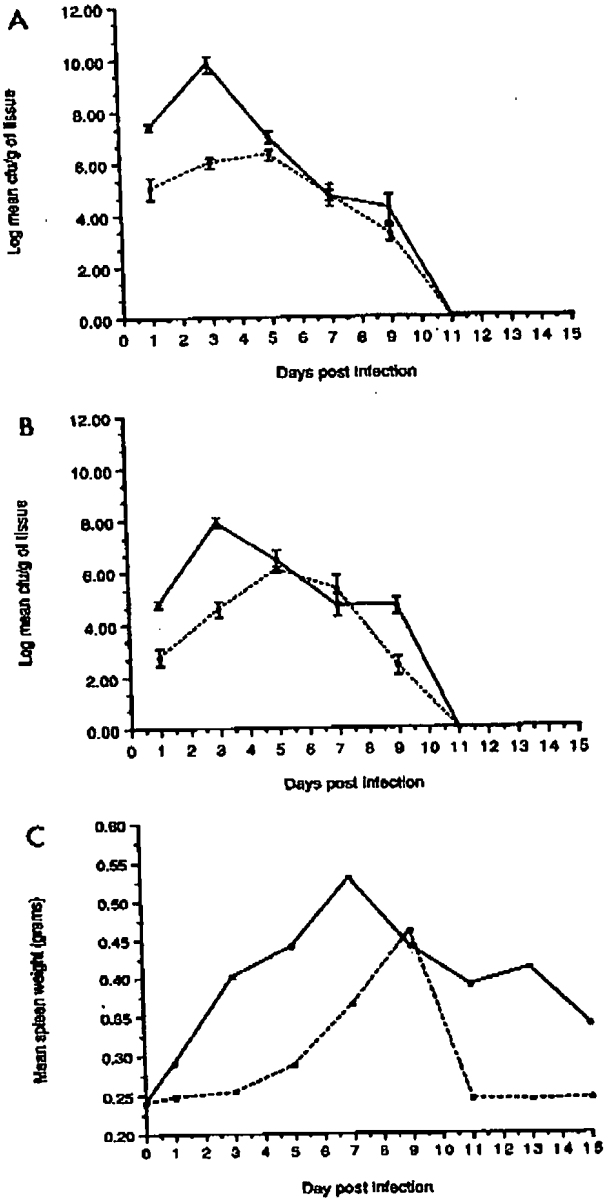

FIG. 2. (A and B) Concentrations of viable Lm.1070138 (——) and Lm.918.6 (– – –) in spleens (A) and livers (B) of MP1 mice over 15 days after intravenous infection with 10^4 CFU. Each point (A and B) represents the geometric mean for five mice. (C) Splenomegaly (expressed as mean spleen weight, in grams) induced over 15 days by infection with 10^4 CFU of Lm.1070138 (——) and Lm.918.6 (– – –). Each point represents the arithmetic mean for five mice.

TABLE 2. Effect of vaccination with Lm.918.6 on the ED_{50} of wild-type Lm.1070138 in mice

Lm.918.6 vaccination[a]	Lm.1070138 ED_{50} (CFU)		
	Mean[b]	SEM	95% CL[c]
None	5.51×10^4	4.79×10^2	1.28×10^4–2.37×10^5
1 dose	$1.73 \times 10^{6*}$	5.35×10^3	1.73×10^5–8.82×10^6
2 doses	$6.00 \times 10^{6**}$	6.09×10^3	1.37×10^6–2.62×10^7

[a] Each vaccinating dose contained 10^8 CFU of Lm.918.6, given intravenously.
[b] Mean dose required to render 50% of the mice moribund. *, Significantly different ($P < 0.05$) from value for unvaccinated mice; **, significantly different ($P < 0.05$) from value for mice receiving one dose.
[c] CL, confidence limits.

different ($P > 0.05$) from that induced by Lm.1070138 (Fig. 2C), but the duration was significantly reduced, with spleens returning to normal weight 11 days postinfection (Fig. 2C). In contrast, with Lm.1070138, splenomegaly was maintained for at least 15 days postinfection (Fig. 2C). In all these experiments, the phenotype and genotype of Lm.918.6 recovered from the infected animals were confirmed by growth on minimal medium and by Southern blotting with Tn*917*-specific DNA probes. In all cases, Lm.918.6 remained $PheA^-$ with a single transposon insertion (data not shown).

To determine whether vaccination with Lm.918.6 conferred protection against subsequent infection, mice were first vaccinated with Lm.918.6 and then challenged 28 days later with Lm.1070138. The ED_{50} of Lm.1070138 was significantly ($P < 0.05$) greater in mice vaccinated with one dose of Lm.918.6 than in nonvaccinated mice (Table 2). In addition, a significant ($P < 0.05$) booster effect was seen when mice were given a second dose of Lm.918.6 14 days after the initial vaccination (Table 2). Therefore, strain Lm.918.6 with a transposon insertion which affects *pheA* expression is attenuated in the mouse model for listeriosis. In the same system, it confers significant protection against infection by virulent *L. monocytogenes*, with a pronounced booster effect upon second vaccination. This is the first evidence that an auxotrophic mutant of *L. monocytogenes* can be attenuated in a manner similar to that described for *aro* mutants of *S. typhimurium* (17). Why a marked reduction in *pheA* gene expression should result in attenuation is not yet clear. It has been hypothesized that in *aroA* mutants of *S. typhimurium* attenuation may be due to an inability to synthesize *p*-aminobenzoate and dihydroxybenzoate (7). Since Lm.918.6 should be capable of making both *p*-aminobenzoate and dihydroxybenzoate, attenuation in this case may reflect the poor availability of phenylalanine in mammalian cells. In addition, feedback inhibition of earlier aromatic pathway enzymes by the accumulation of prephenate, the substrate of prephenate dehydratase (9), may also be occurring. Reduced virulence of *pheA* mutants of gram-negative or gram-positive bacteria has not, to our knowledge, been reported previously. Studies of *aro* mutants of *Salmonella* spp. (4, 11) suggest that a strain of *L. monocytogenes* with multiple lesions in the pathway of aromatic amino acid synthesis might prove to be more attenuated than Lm.918.6. Future studies will attempt to introduce such additional mutations into Lm.918.6. This should serve to increase the attenuation and also reduce the risk of possible reversions in vivo. It is entirely feasible that such strains could be useful as vaccines in farm animals.

In addition to their potential as vaccine strains, nonreverting aromatic amino acid-dependent mutants of *L. monocytogenes* may be exploited as carriers of cloned gram-positive virulence factors and protective antigens of other pathogens, as has been reported for similar strains of *Salmonella* (4, 14).

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Insertional Mutagenesis of *Listeria monocytogenes* with a Novel Tn*917* Derivative That Allows Direct Cloning of DNA Flanking Transposon Insertions

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To carry out efficient insertional mutagenesis in *Listeria monocytogenes* and to facilitate the characterization of disrupted genes, two novel derivatives of Tn*917* were constructed, Tn*917*-LTV1 and Tn*917*-LTV3. The derivatives (i) transpose at a significantly elevated frequency, (ii) generate transcriptional *lacZ* fusions when inserted into a chromosomal gene in the appropriate orientation, and (iii) allow the rapid cloning in *Escherichia coli* of chromosomal DNA flanking transposon insertions. The rapid cloning of DNA flanking insertions is possible because the transposon derivatives carry ColE1 replication functions, a cluster of polylinker cloning sites, and antibiotic resistance genes selectable in *E. coli* (*bla* in the case of Tn*917*-LTV1; *neo* and *ble* in the case of Tn*917*-LTV3). The enhanced transposition frequency of Tn*917*-LTV1 and Tn*917*-LTV3 (about 100-fold in *Bacillus subtilis*) is believed to be due to the fortuitous placement of vector-derived promoters upstream from the Tn*917* transposase gene. In *L. monocytogenes*, Tn*917*-LTV3 transposed at a frequency of 8×10^{-4} when introduced on a pE194Ts-derived vector and generated at least eight different auxotrophic mutations. Two nonhemolytic insertion mutants of *L. monocytogenes* were isolated, and DNA flanking the transposon insertions was cloned directly into *E. coli*, making use of the ColE1 *rep* functions and *neo* gene carried by Tn*917*-LTV3. Both insertions were shown to be within *hlyA*, the *L. monocytogenes* hemolysin structural gene. Although Tn*917*-LTV1 and Tn*917*-LTV3 were constructed specifically for genetic analysis of *L. monocytogenes*, their enhanced transposition frequency and convenience for cloning of DNA adjacent to sites of insertions make them the transposon derivatives of choice for insertional mutagenesis in any gram-positive bacteria that support replication of pE194Ts.

Listeria monocytogenes is a gram-positive, facultative intracellular pathogen responsible for infrequent but often severe infections in humans characterized by meningitis, meningoencephalitis, septicimia, and fetal death (37). *L. monocytogenes* is ubiquitous in nature and, in addition, can be isolated from a wide variety of warm-blooded animals (36). Historically, *L. monocytogenes* has been used as a model intracellular pathogen for studies of cell-mediated immunity (18, 25, 30). Recently, it has been shown that *L. monocytogenes* can infect and grow within a wide variety of cultured animal cells (16, 23, 33) and spread cell-to-cell without ever leaving the host cytoplasm (39). Very little is understood, however, about bacterial determinants necessary for cell attachment, uptake, intracellular growth, or cell-to-cell spread during the course of an infection. Such studies have been hindered by the lack of tools for genetic manipulation of *L. monocytogenes*.

Transposon-mediated insertional mutagenesis was recently demonstrated in *L. monocytogenes* by the introduction of the conjugative transposons Tn*1545* (11) and Tn*916* (15). Tn*1545* was delivered into *L. monocytogenes* at a frequency of approximately 10^{-8} through conjugation with strains of *L. monocytogenes* harboring this transposon (17), and Tn*916* was introduced through conjugation with Tn*916*-containing strains of *Enterococcus faecalis* at a frequency of 10^{-6} (20). These relatively low frequencies of transposition make it inconvenient to carry out large-scale mutagenesis, however, and the randomness of insertion of conjugative transposons is limited by the requirement for sequence homology between both ends of the elements and sequences surrounding the sites of integration (9, 35). Cossart et al. (10) have recently introduced the Tn*3*-like transposon Tn*917* into *L. monocytogenes*, carried on vector pTV1, and demonstrated its utility for insertional mutagenesis. This transposon exhibits a high degree of insertional randomness in *Bacillus subtilis* as well as many other gram-positive bacteria and generates extremely stable insertional mutations (47, 49). More importantly, extensive information exists concerning the physical and genetic organization of Tn*917*, which facilitates altering the transposon in ways that might enhance its utility in species such as *L. monocytogenes* (38, 48).

To take full advantage of the fact that Tn*917* can function in *L. monocytogenes*, we have constructed two modified forms of the transposon, Tn*917*-LTV1 and Tn*917*-LTV3. These derivatives were designed to include the following features. First, they are carried by highly temperature-sensitive derivatives of vector pE194Ts (41). This simplifies the recovery of chromosomal insertions. Second, they contain a promoterless copy of the *Escherichia coli lacZ* gene orientated such that insertions into chromosomal genes can generate transcriptional *lacZ* fusions. Third, they contain, immediately downstream from the *lacZ* coding sequence, an *E. coli* cloning vector that includes a gene selectable in *E. coli*, a gene selectable in *B. subtilis*, ColE1 replication functions, an M13 origin of replication, and a cluster of polylinker cloning sites. The polylinker sites facilitate the recovery in *E. coli* of chromosomal DNA adjacent to sites of insertion, particularly DNA on the promoter-proximal side of transposon-mediated *lacZ* fusions. An extremely impor-

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tant but unanticipated advantage of these transposon derivatives is the fact that they exhibit a much higher frequency of transposition than previously described versions of Tn*917* (as much as 100-fold greater in *B. subtilis*). This should greatly simplify obtaining libraries of transposon-mediated *lac* fusions in a wide range of bacteria.

To investigate the randomness of Tn*917* insertions in *L. monocytogenes* and to test the utility of our modified derivatives of the transposon, several independent insertion libraries were obtained with Tn*917*-LTV3 and screened for various kinds of insertional mutations. Insertional auxotrophic mutations in at least eight distinct loci were obtained. The results suggest that hotspots may exist in the *L. monocytogenes* chromosome where Tn*917* insertions are more frequent but that the overall degree of randomness is of a high order. Several insertions were also obtained within the *hlyA* gene, which encodes a hemolysin protein whose activity is a critical virulence determinant of the organism. A physical analysis of these *hlyA*::Tn*917*-LTV3 insertions revealed them to be distributed throughout the *hlyA* coding sequence and in both orientations with respect to the transcriptional polarity of the gene. Insertions in the appropriate orientation expressed β-galactosidase at high levels. Two of the insertions were used to rescue, into *E. coli*, chromosomal DNA flanking the insertion junctions.

MATERIALS AND METHODS

Bacterial strains. The *B. subtilis* host for plasmid constructions and transposition studies was BD170 (*trpC2 thr-5*) (13). The *E. coli* host for plasmid constructions was HB101 [*hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20*(Sm^r) *xyl-5 mtl-1 supE44* F^- λ^-] (5). The *E. coli* host for recovery of *L. monocytogenes* DNA sequences flanking transposon insertions was MC1061 [*hsdR mcrB araD139* Δ(*araABC-leu*)*7679* Δ*lacX74 galU galK rpsL thi*] (7). The *L. monocytogenes* host for transposition studies was 10403S (2).

Culture media and reagents. Unless otherwise specified, all strains were cultured on Luria-Bertani (LB) medium (12). All antibiotics were purchased from Sigma and were used at the following concentrations: 12.5 μg of tetracycline per ml, 1 μg of erythromycin per ml, 25 μg of lincomycin per ml, 10 μg of chloramphenicol per ml, 50 μg of ampicillin per ml, and 20 μg of kanamycin per ml. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was used at 40 μg/ml in agar media. β-Galactosidase activity in LB liquid cultures was assayed as described by Miller (28). All restriction enzymes and DNA-modifying enzymes were used according to the specifications of the manufacturer.

Construction of pLTV1 and pLTV3. The extreme replication thermosensitivity of pE194Ts is due to a point mutation in its *repC* gene (41). This mutation was transferred to pTV51 (50) by transforming *B. subtilis* PY339 containing pBD95Ts (46) to Tc^r with pTV51 DNA linearized by digestion with *Pst*I. Among the in vivo products of recombination was pTV51Ts, whose temperature-sensitive replication properties were evaluated on media containing erythromycin and lincomycin. To obtain pLTV1 (Fig. 1), equimolar quantities of *Bam*HI-digested pTV51Ts and *Hin*dIII-digested pBG5 DNA (50) were ligated at a total DNA concentration of 10 μg/ml, after treating digested fragments with the Klenow fragment of DNA polymerase I to produce flush ends (43), and the ligation products were used to transform *E. coli* HB101 to Tc^r by using standard methods (34). To obtain pLTV3, the *bla* gene in pBG5 was replaced with the *neo* and *ble* genes from Tn*5* (26) prior to insertion into Tn*917-lac*. This was accomplished by insertion of the 1.9-kilobase *Hin*dIII-*Bam*HI fragment from Tn*5* containing its *neo* and *ble* genes into the 4.5-kilobase *Pvu*I-*Eco*RI backbone of pBG5, after treatment of fragments to produce flush ends (43). To recover pBG5-*neo*, the ligation mixture was used to transform *E. coli* HB101 and Km^r transformants containing pBG5-*neo* were selected. Plasmid pBG5-*neo* was digested with *Hin*dIII, treated with Klenow fragment, and ligated in the presence of an equimolar amount of *Sma*I-digested pTV51Ts. To recover pLTV3, the ligation mixture was used to transform *E. coli* HB101, with selection for Tc^r transformants. Plasmid DNA was prepared from *E. coli* HB101 transformants harboring pLTV1 and pLTV3 after growth in LB (12) containing ampicillin (pLTV1) or kanamycin (pLTV3).

Plasmids pLTV1 and pLTV3 were introduced into *B. subtilis* BD170 by transformation of naturally competent cells (1). *B. subtilis* transformants containing pLTV1 and pLTV3 were designated strains DP-B982 and DP-B983, respectively. Plasmid pLTV3 was introduced into *L. monocytogenes* 10403S by transformation of protoplasts as described below.

Transformation of *L. monocytogenes*. Protoplasts of *L. monocytogenes* were prepared and transformed with plasmid DNA by using a modification of previously described procedures (42). Log-phase *L. monocytogenes* cells at an optical density (600 nm) of 0.8 were harvested from a 20-ml brain heart infusion culture and were washed in 20 ml of H_2O. The cells were suspended in 2 ml of 0.1 M sodium phosphate (pH 7.0), and 0.5-ml portions were dispensed into four sterile 15-ml Corex tubes. To each tube, 4.5 ml of a solution containing 0.67 M sodium phosphate (pH 7.0) and 0.34 mg of bile salts (Sigma) per ml was then added. A 1 mg/ml solution of porcine pancrease lipase (Sigma) in 0.1 M sodium phosphate (pH 7.0) was centrifuged at 10,000 × *g* in a microcentrifuge for 30 s, and 1, 3, 5, or 10 μl of the clear supernatant was added to each tube of cells. The cells were incubated at 37°C with gentle shaking for 15 min, 50 μl of 0.1 M $CaCl_2$ was added to each tube, and the incubation was continued for 45 min with gentle shaking. A 3-ml portion of 0.155 M NaCl was added to each tube, and the cells were pelleted at 4,300 × *g* for 15 min. Each cell pellet was washed once with 5 ml of 0.155 M NaCl and suspended in 0.5 ml of 0.155 M NaCl by vortexing vigorously. To each tube, 4.4 ml of a solution containing 0.34 mg of lysozyme per ml, 0.03 M Tris hydrochloride (pH 6.7), and 0.45 M sucrose was then added. The cells were incubated at 37°C for 15 min with gentle shaking, 0.1 ml of 1 M $MgCl_2$ was added to each tube, and the incubation was continued for 3 h at 37°C with gentle shaking. Formation of protoplasts was followed by phase-contrast microscopy. Cells from the tube showing the highest percentage of protoplasts were pelleted at 7,600 × *g* at 25°C, washed once in 5 ml of SMMP (2× Difco antibiotic medium no. 3, 0.5 M sucrose, 0.02 M maleate, 0.02 M $MgCl_2$ [pH 6.5], adjusted with NaOH), and suspended in 200 μl of SMMP. For transformation, 1 μg of pLTV3 DNA was added and the solution was mixed by gentle swirling followed by the addition of 600 μl of 40% PEG (Sigma; M_w 2,500) in 2× SMM (SMM is SMMP without Difco medium). The solution was gently mixed by swirling, and after 3 min at room temperature, 6 ml of SMMP was added. The protoplasts were pelleted at 7,600 × *g* for 15 min at 25°C, resuspended in 200 μl of SMMP containing a subinhibitory but inducing concentration of erythromycin (0.04 μg/ml), and incubated at 30°C for 1 h to allow the inducible expression of the

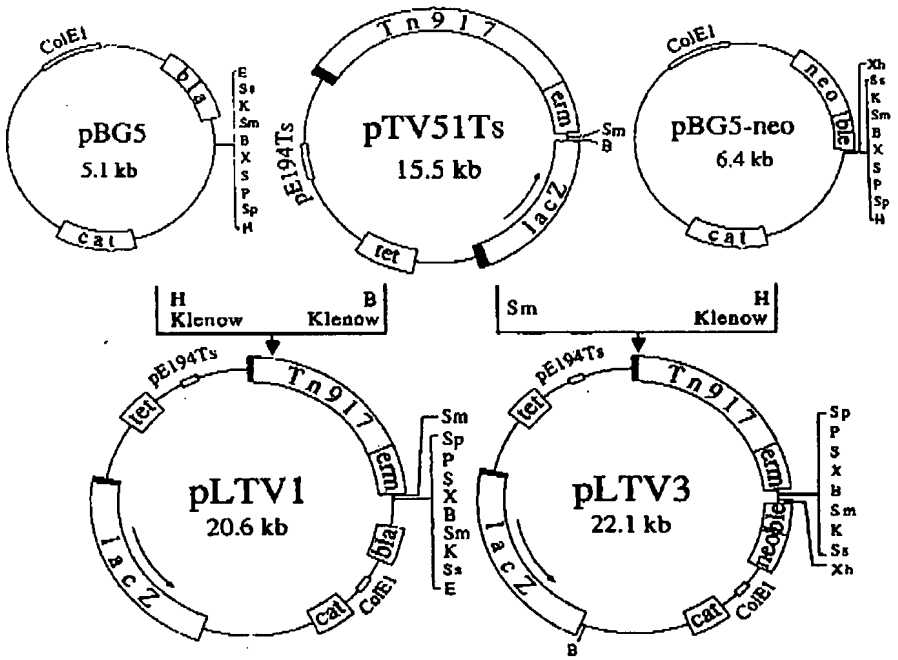

FIG. 1. The construction of pLTV1 and pLTV3. Plasmid pBG5 (50) is a ColE1-derived replicon which contains the pBR322 β-lactamase gene (*bla*) (4), M13mp19 polylinker (45), and *Staphylococcus aureus* pC194-derived chloramphenicol acetyltransferase gene (*cat*) (19). Plasmid pBG5-*neo* contains the neomycin phosphotransferase II (*neo*) and bleomycin (*ble*) determinants of Tn5 (26) in place of the *bla* gene of pBG5. Plasmid pTV51Ts contains the pE194Ts temperature-sensitive replicon (41), the tetracycline resistance gene (*tet*) from pAMα1Δ1 (31), and Tn*917-lac* (32), which contains a promoterless *lacZ* gene from *E. coli* with translation initiation signals derived from *B. subtilis* gene *spoVG* (51) and Tn*917* ribosomal methytransferase gene (*erm*). Klenow refers to treatment of restriction fragments with the large subtilisn-generated fragment of *E. coli* DNA polymerase I (21) in the presence of deoxyribonucleoside triphosphates to produce blunt ends (43). Restriction endonuclease abbreviations used: *Eco*RI (E), *Sst*I (Ss), *Kpn*I (K), *Sma*I (Sm), *Bam*HI (B), *Xba*I (X), *Sal*I (S), *Pst*I (P), *Sph*I (Sp), *Hin*dIII (H), and *Xho*I (Xh).

transposon-encoded *erm* gene. Protoplasts were plated onto DM3 plates (8) (0.5 M sodium succinate [pH 7], 0.5% Casamino Acids, 0.5% yeast extract, 0.35% K_2HPO_4, 0.15% KH_2PO_4, 0.5% glucose, 0.02 M $MgCl_2$, 0.01% bovine serum albumin, 0.8% agar) containing 1 μg of erythromycin per ml and incubated at 30°C. Erythromycin-resistant transformants containing pLTV3 formed small L-form colonies (22) after 2 days and large colonies, in which the bacterial cell walls had regenerated, after 3 to 4 days. An *L. monocytogenes* transformant containing pLTV3 was designated strain DP-L910. All unspecified reagents used were from Sigma.

Determination of transposition frequencies. Single colonies of *B. subtilis* DP-B982(pLTV1), DP-B983(pLTV3), or *L. monocytogenes* DP-L910(pLTV3) were picked after overnight growth at 30°C on LB agar containing erythromycin, lincomycin, and tetracycline and used to inoculate 10 ml of LB cultures containing the above antibiotics. *B. subtilis* PY313 containing pTV1Ts (46) was also grown overnight and used to inoculate a culture as above, except that chloramphenicol was used in place of tetracycline. All four cultures were grown with aeration at 30°C to an optical density (600 nm) of approximately 0.4. Dilutions of each culture were made, and the number of CFU at the permissive (30°C) and nonpermissive (47°C for *B. subtilis* and 41°C for *L. monocytogenes*) temperatures for plasmid replication were determined on LB agar containing erythromycin and lincomycin. The transposition frequencies were calculated by dividing the titers at the nonpermissive temperatures by those at 30°C. Transposition frequency determinations were performed in triplicate for each strain, and the frequencies were then averaged.

Isolation of transposon insertions. A single colony of *L. monocytogenes* DP-L910(pLTV3) was used to inoculate 2 ml of brain heart infusion containing erythromycin, lincomycin, and tetracycline, and the culture was grown overnight at 30°C to stationary phase. The overnight culture was inoculated 1/800 into brain heart infusion containing erythromycin and lincomycin, and the bacteria containing chromosomal transposon insertions were selected for by growth with aeration at 41°C until stationary phase. This treatment resulted in a population of cells of which 90% were Em^r, Lm^r, and Tc^s, indicating loss of pLTV3 with retention of transposon insertions into the chromosome. Aliquots of the culture were directly frozen in LB broth at −70°C until later use. The frozen aliquots from a single culture will be referred to as transposon insertion libraries of *L. monocytogenes*.

Characterization of insertional mutants. A total of 1,000 colonies of *L. monocytogenes* from each of 10 separate transposon insertion libraries were patched onto minimal medium (44) to screen for 18 common auxotrophic types. Mutant strains unable to grow on the minimal media were subsequently analyzed to determine their specific auxo-

trophic requirements, as described by Davis et al. (12). Nonhemolytic transposon insertion mutants of *L. monocytogenes* were isolated by plating insertion libraries directly onto blood agar and screening for colonies lacking a zone of hemolysis.

Cloning of DNA flanking transposon insertions. Chromosomal DNA from transposon insertion mutants of *L. monocytogenes* was isolated as described by Flamm et al. (14). Chromosomal DNA was digested with *Xba*I, followed by ligation in a 100-μl volume at a DNA concentration of 5 μg/ml. The ligated DNA was concentrated by ethanol precipitation and used to transform *E. coli* HB101 to Km^r. Plasmid DNA was isolated from Km^r transformants and analyzed by restriction enzyme analysis.

Southern blot analysis. Southern blot analysis of transposon insertions in the *L. monocytogenes* hemolysin structural gene, *hlyA* (27), was done as previously described (6).

RESULTS

Construction of pLTV1 and pLTV3. In previous work (48), it was shown that insertion of foreign DNA into a particular region of Tn*917* near the *erm*-proximal end did not affect transposition. Making use of two unique restriction sites present within this region of the transposon derivative in pTV51Ts, linearized pBG5 and pBG5-*neo* were inserted to create pLTV1 and pLTV3, respectively (Fig. 1). The Tn*917*-derivatives in pLTV1 and pLTV3 were designated Tn*917*-LTV1 and Tn*917*-LTV3, respectively. The structures and functional properties of pLTV1 and pLTV3 were confirmed by the following criteria: restriction enzyme analysis (data not shown); ability to confer resistance to tetracycline and ampicillin (pLTV1) or tetracycline and kanamycin (pLTV3) in *E. coli*; ability to confer resistance to erythromycin, lincomycin, tetracycline, and chloramphenicol in *B. subtilis* and *L. monocytogenes*; and ability of *B. subtilis* strains containing pLTV1 or pLTV3 to form blue colonies on LB agar containing 40 μg of X-gal per ml (29). The parental *L. monocytogenes* 10403S had a low endogenous β-galactosidase activity, forming very light blue colonies on LB agar after 48 h of growth at 30°C. However, many transposon insertions into transcriptionally active regions of the *L. monocytogenes* chromosome were easily detected above this background, causing colonies containing them to turn dark blue on X-gal plates. When subsequently examined in liquid culture, some of these insertions were found to produce greater than 1,000-fold more β-galactosidase activity than the wild type.

To determine whether the insertion of pBG5 or pBG5-*neo* into Tn*917-lac* affected transposition, the transposition frequencies of Tn*917-LTV1* and Tn*917*-LTV3 in *B. subtilis* were determined and compared with an unaltered version of Tn*917* (Table 1). The transposition frequency of Tn*917* in *B. subtilis* was 5.8×10^{-5}, which was similar to that previously reported (47). Surprisingly, the transposition frequency of Tn*917*-LTV1 and Tn*917*-LTV3 in *B. subtilis* was approximately 100-fold greater. Transposition of Tn*917*-LTV3 in *L. monocytogenes* was approximately sevenfold less frequent than in *B. subtilis*, which was similar to that previously reported for Tn*917* (10).

Randomness of transposon insertion in *L. monocytogenes*. To evaluate the randomness of Tn*917* insertions in the *L. monocytogenes* chromosome, 10,000 insertions (1,000 from each of 10 independent libraries) were characterized to determine whether they included different kinds of auxotrophic mutations. Insertional auxotrophic mutants were isolated at a frequency of 0.82%. Among the 82 auxotrophs isolated, the following common requirements were found: 48 adenosine, 14 uracil, 8 proline, 5 glycine, 3 nicotinic acid, 2 phenylalanine, 1 glutamine, and 1 aromatic amino acids (Table 2). Wild-type *L. monocytogenes* strains require cysteine, glutamine, isoleucine, valine, arginine, histidine, methionine, tryptophan, thioctic acid, riboflavin, thiamine, and biotin for growth on a synthetic minimal medium, which would have prevented the detection of several common auxotrophic types (44). Thus, we obtained 8 of 18 possible common auxotrophic types screened for, suggesting that Tn*917*-LTV3 can insert into the *L. monocytogenes* chromosome with a relatively high degree of randomness. Nevertheless, as in *B. subtilis*, there would appear to be hotspot regions of the chromosome where insertions are more abundant (49). At least one of these hotspot regions apparently contains genes required for adenine biosynthesis.

To evaluate the insertional randomness within a single chromosomal locus in *L. monocytogenes*, we isolated insertions in the listeriolysin O structural gene, *hlyA* (27), that caused a nonhemolytic phenotype. Nonhemolytic insertion mutants were detected at a frequency of 6×10^{-3}. Eight independent insertions were mapped within *hlyA*, of which three formed blue colonies on LB agar containing X-gal and had β-galactosidase activities in solution 1,000-fold higher than the wild type. As hemolysin is strongly expressed on LB agar (A. Camilli and D. Portnoy, unpublished data), this suggested that transcriptional fusions between *hlyA* and the transposon-containing *lacZ* gene had formed in these three mutants. The approximate sites of insertion and the transposon orientations of the eight *hlyA* insertions are shown in Fig. 2.

TABLE 1. Transposition frequencies

Bacteria	Transposon (vector)[a]	Transposition frequency[b]
B. subtilis	Tn*917* (pTV1)	5.8×10^{-5} (± 5.0)
B. subtilis	Tn*917*-LTV1 (pLTV1)	5.5×10^{-3} (± 1.7)
B. subtilis	Tn*917*-LTV3 (pLTV3)	6.1×10^{-3} (± 4.6)
L. monocytogenes	Tn*917*-LTV3 (pLTV3)	8.2×10^{-4} (± 2.5)
B. subtilis	Tn*917-lac* (pTV51Ts)	2.0×10^{-4}[c]
B. subtilis	Tn*917-lac* (pTV32Ts)	8.0×10^{-5}[c]

[a] Vector refers to the plasmid on which each transposon was carried prior to transposition into the host chromosome.
[b] Mean and standard deviation of three separate determinations.
[c] One determination.

TABLE 2. Auxotrophic types

Transposon library	Auxotrophs isolated[a]
1	1 Ade, 1 Gly, 2 Pro, 1 Ura
2	3 Ade, 2 Ura
3	2 Ade, 1 Aro, 2 Gly, 1 Nic, 1 Phe, 1 Ura
4	9 Ade, 1 Gln, 1 Gly, 2 Pro, 1 Ura
5	6 Ade, 1 Ura
6	1 Ade, 1 Nic, 3 Ura
7	7 Ade, 2 Pro, 1 Ura
8	10 Ade, 1 Nic, 1 Phe, 1 Ura
9	5 Ade, 1 Pro, 1 Ura
10	4 Ade, 1 Gly, 1 Pro, 2 Ura

[a] Abbreviations: adenosine (Ade), aromatic early block (Aro), glutamine (Gln), glycine (Gly), nicotinic acid (Nic), phenylalanine (Phe), proline (Pro), and uracil (Ura).

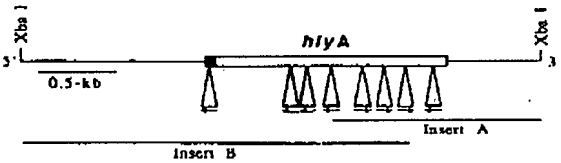

FIG. 2. Physical map of *L. monocytogenes hlyA* chromosomal region showing sites of Tn*917*-LTV3 insertion and cloned flanking DNA. Only sites for *Xba*I are shown. The solid portion of *hlyA* represents the predicted signal sequence (27). Locations of Tn*917*-LTV3 insertions are indicated below *hlyA* by open triangles. The transcriptional orientations of the transposon-associated *lacZ* gene are indicated by horizontal arrows below each insertion. The locations of cloned flanking sequences (insert sequences A and B) from two transposon insertions are shown below *hlyA*. Insert A was obtained, after digestion with *Xba*I, from the transposon insertion directly above the 5' end of insert A. Insert B was similarly obtained from the transposon insertion directly above its 3' end. Mapping was based on single and double digestions of the cloned flanking sequences with the appropriate restriction enzymes (data not shown), on Southern blot analysis of wild-type *L. monocytogenes* chromosomal DNA digests probed with labeled inserts A and B (data not shown), and on the published *hlyA* sequence (27).

Direct cloning of DNA flanking transposon insertions. To demonstrate that chromosomal DNA flanking the sites of transposon insertions could be cloned directly into *E. coli* by using the ColE1 replicon within Tn*917*-LTV3, we cloned flanking DNA from two *hlyA* insertions. Taking advantage of known restriction sites in the vicinity of *hlyA*, DNA flanking the insertions was easily cloned by using the restriction enzyme *Xba*I. Because an *Xba*I site is present in the polylinker of Tn*917*-LTV3, sequences cloned by using *Xba*I digests extended in one direction only, from the site of insertion (Fig. 2 and 3).

DISCUSSION

To facilitate the study of bacterial determinants of *Listeria monocytogenes* pathogenicity, we have constructed transposition-proficient derivatives of Tn*917-lac* containing ColE1 replication functions. By using one of these derivatives (Tn*917*-LTV3), we have shown that Tn*917* can insert into the *L. monocytogenes* chromosome with a relatively high degree of randomness, generating *lacZ* transcriptional fusions when insertions occur within genes in the appropriate orientation. The presence of ColE1 replication functions and polylinker cloning sites allowed the convenient and rapid cloning of flanking DNA. Unexpectedly, both derivatives, Tn*917*-LTV1 and Tn*917*-LTV3, exhibited enhanced transposition frequencies in *B. subtilis*. Although the reason for the increased transposition frequencies was not determined, it is possible that the level of expression of the Tn*917* transposase is increased in these constructs due to transcriptional readthrough from within the inserted ColE1 replicons. In the case Tn*917*-LTV1, the *lac* promoter adjacent to the polylinker cluster in pBG5 (50) is positioned appropriately to direct transcription toward the Tn*917* transposase gene. This promoter is deleted in Tn*917*-LTV3 but is replaced by the promoter for the Tn*5* Sm^r gene (26). The increased frequencies of transposition of pLTV1 and pLTV3 should greatly facilitate their use for insertional mutagenesis in *B. subtilis*, and potentially in other gram-positive bacteria as well, by reducing the culture volumes necessary to produce transposon insertion libraries.

Transposons Tn*917*-LTV1 and Tn*917*-LTV3 differ only in the gram-negative antibiotic resistance marker contained within their ColE1-derived sequences. Although we would expect that the gram-negative *bla* gene present in Tn*917*-LTV1 would not confer resistance to ampicillin in *L. monocytogenes*, we considered it undesirable to introduce this gene into a pathogen for which β-lactam antibiotics are clinically important therapeutics. Thus, we replaced the *bla* gene with the *neo* and *ble* genes from Tn*5* in the construction of Tn*917*-LTV3. Although Tn*917*-LTV3 might be the transposon of choice for other gram-positive pathogens as well (e.g., *Streptococcus* spp.), Tn*917*-LTV1 should prove generally more useful for insertional mutagenesis in nonpathogenic gram-positive species since it contains additional unique restriction sites within its polylinker region. Schematic examples of *lacZ* fusions mediated by Tn*917*-LTV1 and Tn*917*-LTV3 are shown in Fig. 3 to illustrate the kinds of restriction sites available in both transposon derivatives for the cloning of DNA flanking transposon insertions.

The randomness of Tn*917* insertions into chromosomal targets has been most extensively investigated in *B. subtilis* (40) and *B. megaterium* (3), although the transposon has been shown to function efficiently in a broad range of bacteria, including both gram-positive and gram-negative species (24). Particularly in *B. subtilis*, it would appear that insertions in some hotspot chromosomal regions are much more frequent than in others (49). Even within these hotspot regions, however, insertions are distributed quite randomly, and insertions outside of hotspot regions are sufficiently abundant and random to permit very effective insertional mutagenesis (40, 49). Our results suggest that the same is true for *L. monocytogenes*. Although the distribution of insertional auxotrophic mutations was not completely random, many different kinds were recovered, even in this relatively limited study. Insertions within the *hlyA* gene are significantly more frequent than would be expected on a purely random basis, suggesting that this gene may be within

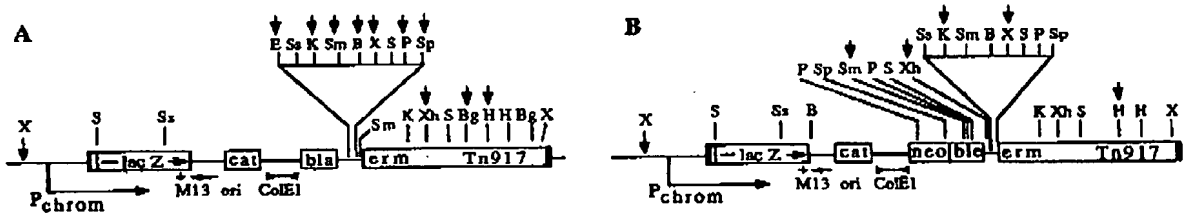

FIG. 3. Hypothetical chromosomal insertions of Tn*917*-LTV1 (A) and Tn*917*-LTV3 (B). In both cases, a transcriptional fusion between the transposon-containing *lacZ* gene and a chromosomal promoter is shown. Unique sites in or near the polylinkers, which can be used to clone adjacent sequence flanking the left end of the transposons, are indicated by vertical arrows. Restriction endonuclease abbreviations used: *Eco*RI (E), *Sst*I (Ss), *Kpn*I (K), *Sma*I (Sm), *Bam*HI (B), *Xba*I (X), *Sal*I (S), *Pst*I (P), *Sph*I (Sp), *Hin*dIII (H), *Xho*I (Xh), and *Bgl*II (Bg).

one of the hotspot regions of the *L. monocytogenes* chromosome. In addition, as in *B. subtilis* and other bacteria (46), Tn*917* appears to insert singly into the *L. monocytogenes* chromosome (data not shown).

Despite the large size of Tn*917*-LTV3 (15.5 kilobases), its ability to (i) insert randomly into the *L. monocytogenes* chromosome, (ii) form transcriptional fusions with *lacZ*, and (iii) allow the direct cloning of DNA adjacent to insertions should ensure its utility in future mutational analysis of *L. monocytogenes*. Similarly, Tn*917*-LTV1, which provides even greater flexibility in the choices of restriction enzymes for cloning adjacent DNA, should prove useful for transposon mutagenesis in a wide range of other gram-positive bacteria and particularly in species where this transposon derivative exhibits the high transposition frequency seen in *B. subtilis*.

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